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Adhesion forces measured between a calcium blocker drug and its receptor in living cells using atomic force microscope

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Abstract **The adhesion force between the tip of an atomic force microscope cantilever derivatized with nimodipine (a calcium blocker, from the dihydropyridine class, currently used in clinical medicine for hypertension) and living cells of *Saccharomyces cerevisiae* (unicellular eukaryotes which portray ultrastructural features characteristic of higher eukaryotic cells) was measured. This methodology allowed us to locate (and visualize) pores on the cell surface which may be responsible for calcium transportation in the living cells. The interaction of the cantilever derivatized with the calcium blocker and a pore, which can be a calcium channel, is more intense than a non-derivatized cantilever and the pore. Outside the pore (on the rest of cell surface), a derivatized or a non-derivatized cantilever has the same pattern of adhesion force. The information obtained with this method is very important for the design of new, more potent and less toxic drugs for pharmacological use.**

Key words: Atomic force microscopy; Haloperidol; Enkephalins; Endorphins; Neurotransmitters; Hormone; Neuroleptic; Radiolabeled drugs; Nifedipine derivative

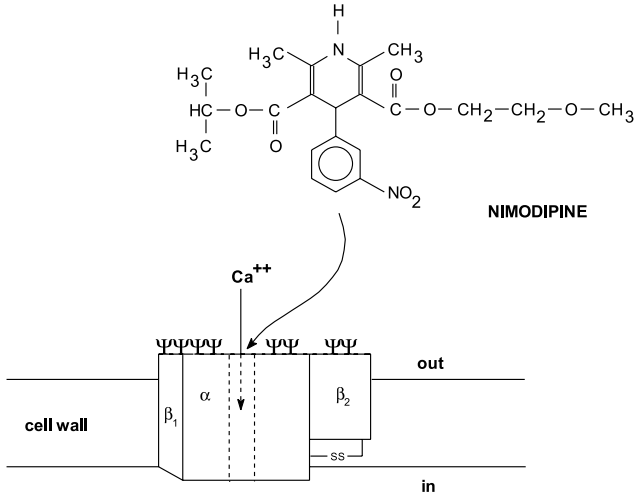
1. Introduction

Saccharomyces cerevisiae has been imaged by atomic force microscopy (AFM) for the last seven years [1–6]. *S. cerevisiae* has cell walls which give a natural rigidity to the sample [7] and, consequently, it is not necessary to use additional methods [1,8] to give a certain rigidity to the membrane of these living cells (sample) [8]. This natural 'fortress' makes it possible to obtain images with these cells in a very natural way, with good resolution and with incredible reproducibility [1,6]. The cell wall protects *S. cerevisiae* against extreme hypotonic media (e.g. ultra-pure water) and these cells do not blow up as do other living cells without cell walls. This property permits the use of ultra-pure water as a reaction medium, in order to avoid interferences of biochemicals (commonly used in reaction medium for equilibrating the osmotic pressure) which could produce artifacts. The experiments made, in this way, are clean, reproducible and reliable [1,6]. For this reason these are the most stable living cells to work with AFM and the images generated are very consistent with those generated by scanning electron microscopy (SEM) in terms of shape and size [9]; with the difference that with AFM these cells are observed alive because the samples are not covered or fixed. So, it is possible to visualize pores on the cell surface, which is impossible with SEM [1,2].

The pores present on the cell wall surface are channels responsible for nutrient and ion transportation to the inside of the living cells. Some of them are responsible for transporting calcium ions. In human cells, the influx of calcium ions regulates the contraction of muscles which can provoke vasoconstriction and an increase of blood pressure. A calcium blocker drug slows down the influx of calcium ions, avoiding vasoconstriction and regulating blood pressure as a consequence [10].

It is known that *S. cerevisiae* cells have calcium channels [11]. These channels, in human cells, were divided in three subtypes, called L, N and T, based on conductance and sensitivity to voltage [12–14]. Only the type L channel is sensitive to the calcium blocker drugs from the dihydropyridine class (nifedipine, nimodipine, nicardipine, nitrendipine and amlodipine), used for years in clinical medicine [10]. Recently, it was described that *S. cerevisiae* cells have type L calcium channels, sensitive to these drugs, as do human cells, and that the dihydropyridine drugs can regulate the influx of calcium to the inside of *S. cerevisiae* (see Scheme 1) [15].

Probably, such calcium blocker drugs bind to specific receptors in the α-chain, provoking a changing in the three-dimensional structure of the protein and, as a consequence,



Scheme 1. Ca^{2+} channel in the cell wall. The nimodipine molecule and calcium channel are out of proportion.

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block the passage of calcium ions. These receptors are located in the pores responsible for calcium transportation as observed in experiments made before. It was verified that the calcium channel works in hypotonic media in the same way as it does in isotonic media [15].

Although knowledge of the molecular structure of receptors is very recent, the concept that drugs elicit their effects by binding to molecular receptors was formulated more than a century ago [10]. The direct identification of receptors for drugs, hormones, and neurotransmitters by means of radiolabeled ligands began in the mid-1960s. It had become apparent that most receptors have such high affinity for their respective 'ligands' (i.e. neurotransmitter, hormone, or drug) that they become saturated when the ligand concentration is in the nanomolar range. Hence, it was necessary to develop methods for preparing radioactive ligands with specific activities sufficiently high to permit detection of the very small amounts of receptors that are present in body tissues [10].

The use of a cantilever derivatized with drugs can replace this technique of radioactive ligands with the advantages that it is possible to see the area of receptors in just a single cell and it is not necessary to use a radiolabeled drug. The use of a cantilever derivatized with molecules (or macromolecules) is one of the most current uses for AFM in the biological and pharmaceutical areas [4–6,16]. Recently, enzymes immobilized on a cantilever were used to identify biomolecules exiting from or entering into living cells of *S. cerevisiae* in real time [4–6].

The objective of this paper is to use a cantilever derivatized with commercial nimodipine (one of the dihydropyridine family) in order to identify the location of the receptors of a typical calcium blocker drug (scheme 2) and, as a consequence, to identify the pores which are responsible for calcium transportation.

With this knowledge it may now be possible to gain more precise information about how drugs bind to specific receptors and, eventually, to design drugs with highly specific binding characteristics.

2. Materials and methods

2.1. Chemicals

Nimodipine was obtained from Galênica (Campinas, São Paulo, Brazil). The reagents used were of analytical grade. Water was doubly distilled and deionized.

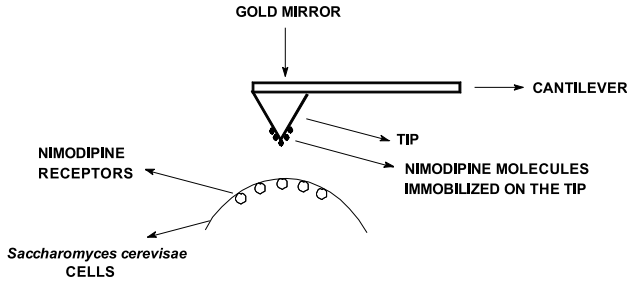
2.2. Biological materials

The industrial strain of *S. cerevisiae* was obtained from Emulzint (USA). The stock suspensions were prepared by adding 1.0 g dry baker's yeast to 10 ml water (double distilled and deionized) while stirring at room temperature. A drop of the suspension was placed on the surface of glass coverslips and allowed to dry for 15–20 min at room temperature to remove the excess water. The experiments were performed under a thin layer of water.

2.3. AFM

An Autoprobe CP AFM (Veeco Instruments) operating in contact mode was used in the experiments with Nanotips (Veeco Instruments), Si_3N_4 tips with 0.12 N/m spring constants were chosen. All images were collected on the AFM using a scan speed of 1.0 Hz, and all imaging was done in air at room temperature.

The AFM works by moving a commercial micro-fabricated tip across a preparation while recording the *X*, *Y* and *Z* coordinates of the preparation being scanned. The *Z* dimension is calculated by having a laser beam reflect off the surface of the cantilever. The laser light reaches the surface of a split photodiode that records changes in the position with high precision. Deflections in the tip, which correspond to the surface topography, are sensed by a photodetector.



Scheme 2. Drawing showing the nimodipine molecules immobilized on the cantilever tip (derivatized cantilever) of AFM, in order to measure the force of interaction between nimodipine and its receptor.

2.4. Cantilever derivatized with nimodipine

1. Stock solution of nimodipine: 100 mM and 10 mg of bovine serum albumin (BSA) were dissolved in 1 ml double distilled and deionized water. 10 μl of this solution (nimodipine plus BSA) were spread on a 2 cm^2 area on a coverslip – according to the modified method described earlier [4].
2. Building the nanobiosensor: a brand new cantilever was put in the cantilever holder of the AFM. A contact was made on a coverslip that contained the spread solution of nimodipine plus BSA. Then 1 μl of 25% glutaraldehyde (crosslinking agent) was added into the area where the solution of nimodipine and BSA was spread. The resultant gel phase covering the cantilever was found to be stable in aqueous solution. After 30 s the cantilever was taken off the coverslip surface and dried for 3 h at room temperature. After this, this derivatized cantilever was put in a vacuum desiccator for storage at 5°C.

The nanobiosensors should be kept in a vacuum and under refrigeration. Under these conditions they can be used up to 4 weeks after preparation and the experiments with them are very reliable and reproducible.

2.5. Adding free nimodipine to the sample

Free nimodipine (300 μM) was added to the cell suspension of *S. cerevisiae* and the mixture was stirred for 3 min. After this period, an aliquot of 10 μl was spread on a 1 cm^2 area on a coverslip. After 15 min the excess water was evaporated, at room temperature, and the experiments of force measurements were made (the first one with a non-modified cantilever and after this with a cantilever derivatized with nimodipine).

3. Results and discussion

3.1. Importance of the AFM for the biological and pharmacological areas

For a very long time, SEMs have been used to obtain high resolution visualizations of the surfaces of biological samples. Normally, in order to scan the samples of *S. cerevisiae* cells, each preparation is coated with a film of evaporated gold (approximately 20 nm in thickness [7,17,18]). The application of this conductive coating to the surface of the sample masks important structures which exist on the cell wall [1,2] and the cells are killed during the coating. With the advent of AFM, this fact changed and the cells are visualized uncoated and alive, at high resolution, producing excellent and reproducible images. Some years ago, the world's first micrographs of different industrial strains of *S. cerevisiae* were generated in an AFM and we could observe structures, on the cell wall, never described before and pores which vary from 50 to 250 nm [1,2].

3.2. The advent of nanobiosensors made with the cantilever of the AFM

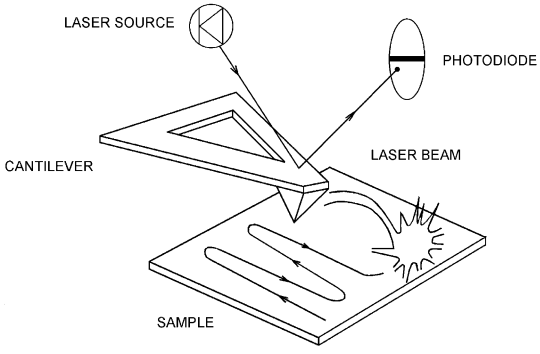
Recently, nanobiosensors were built by immobilizing enzyme molecules on cantilever surface in order to detect movement of biomolecules from inside to outside (and vice versa) of living cells of *S. cerevisiae* [4,5]. When a scanning was made during release of alcohol molecules, from inside of fermenting *S. cerevisiae*, and when these alcohol molecules met the enzyme alcohol dehydrogenase (immobilized on the cantilever surface), a biochemical reaction took place and heat was evolved. Such heat induced a deflection of the cantilever and this was registered on the AFM screen [5].

The deflection of the cantilever is explained by a physical phenomenon when two different metal blades (with, of course, different thermal dilation coefficients) are bonded together. This is called bimetallic system and the presence of heat makes the blade bend. The cantilever of AFM is not a bimetallic blade but can work as one because it has two different materials (a metal and a semi-metal) with different thermal dilation coefficients: gold and silicon (silicon is a semi-metal located in the column IVA of the Periodic Table of the Elements which is a semi-conductor of electricity). If a biochemical (or chemical) reaction takes place near this 'bimetallic blade', it is possible to produce a deflection (bending) in the cantilever. Then, this latter will produce an enormous deviation of the direction of the laser beam which will be recorded in real time by the computer (via a photodiode) [6].

Based on this fact, a new idea was conceived in order to locate important areas on a cell surface: the location of the drugs, hormones, or neurotransmitter receptors using a drug-derivatized cantilever. Some substances (such as calcium blockers and hormones) usually act on the cell surface transmitting signals to the inside of living cells, inducing new biochemical reactions and, sometimes, changing the behavior of the cells [10]. The receptors of such substances are located on unknown areas of the cell surface.

According to the adopted theory of drug action, the drug molecules must insert themselves in the receptors of the organism before these initiate the process of response. So, the intensity of drug action is proportional to the occupancy of the receptors or, in other words, to the concentration of the drug–receptor complexes [10].

So, we could deduce that if a drug (or a hormone which acts on a cell surface) was immobilized on a cantilever tip, it would be possible to locate the above-mentioned receptors via a phenomenon similar to the deflection of a cantilever derivatized with enzyme (nanobiosensor): when a scanning was made and this drug immobilized on the cantilever tip (drug-derivatized cantilever) would meet the protein or enzyme (present on the cell surface, which acts as the receptor of the drug [10]), the Van der Waals forces in this area would be stronger than in any other area of the cell surface. As the scan speed is constant and the cantilever is forced to go out from this area of stronger interaction, a deflection would be observed and registered as a trace on the AFM screen (see Scheme 3). Beyond that, if a force–distance graph was made at the point of the deflection, a stronger Van der Waals force would be observed in relation to the other areas where there are no defletions, indicating that on this small area (defletion area) the receptor of the drug immobilized on the cantilever is located. These assumptions proved to be correct when these experiments were carried out.



Scheme 3. When nimodipine finds its receptor, the Van der Waals forces are stronger in this area. The scan speed of AFM is constant and the cantilever is forced to go out from this area of stronger interaction, which is observed and registered as a trace.

3.3. The use of the nanobiosensor to find calcium channels

At the beginning of the experiment, 1 g of lyophilized *S. cerevisiae* was dissolved in 10 ml of water and stirred at room temperature. A drop of this suspension was placed on a coverslip and dried at 25°C during 15–20 min to remove excess water. Fig. 1A shows the scanning of this sample (ultrapure water+*S. cerevisiae* cells) by a non-modified cantilever (cantilever without drug on its tip) and a known image of these cells was obtained as observed before [1–6]. When the same sample was scanned by a cantilever derivatized with nimodipine (modified cantilever), several traces appeared on the image, indicating that the nimodipine (immobilized on the cantilever tip = modified cantilever) found an area where there could be a calcium channel. When a single cell was chosen and the scanning was repeated on a small area (2 μm), several pores were observed and some traces again appeared. Then, we chose a pore under one of these traces, a force–distance graph was made, and we found that the force on the area of the pore was stronger than outside the pore, indicating that the probable area of the binding (receptor) of the drug is located in the pore and not on the other areas of the cell surface (Fig. 2B). If the same experiment (force–distance curve) is repeated with a non-modified cantilever (without a drug on its tip), the same force is observed inside or outside the pore (Fig. 2A), indicating that the presence of the drug (immobilized on cantilever tip) is necessary to attach to its receptor and to produce an increasing of the force. In Fig. 2C, free nimodipine (300 μM) was added to a suspension of *S. cerevisiae* cells. An aliquot, with this suspension, was taken and placed on a coverslip. The excess water was evaporated as described in Section 2. Probably, with this addition of free nimodipine, the receptors in the cells were totally occupied as deduced in the biochemical experiments made previously [15] where the calcium blockers at 300 μM demonstrated to block any calcium uptake by *S. cerevisiae* cells [15], indicating that the calcium channels were 'paralysed' by the drug action. From this information, it is possible to say that these are specific receptors. These latter are binding sites which can be saturated at the nanomolar concentration (non-specific receptors can not be saturated) [10]. When a new force–distance graph was made, no force was detected, indicating that no interaction of the modified cantilever with the cell surface was observed. This happened, probably, due to the repulsion of the nimodipine molecules immobilized on the cantilever tip

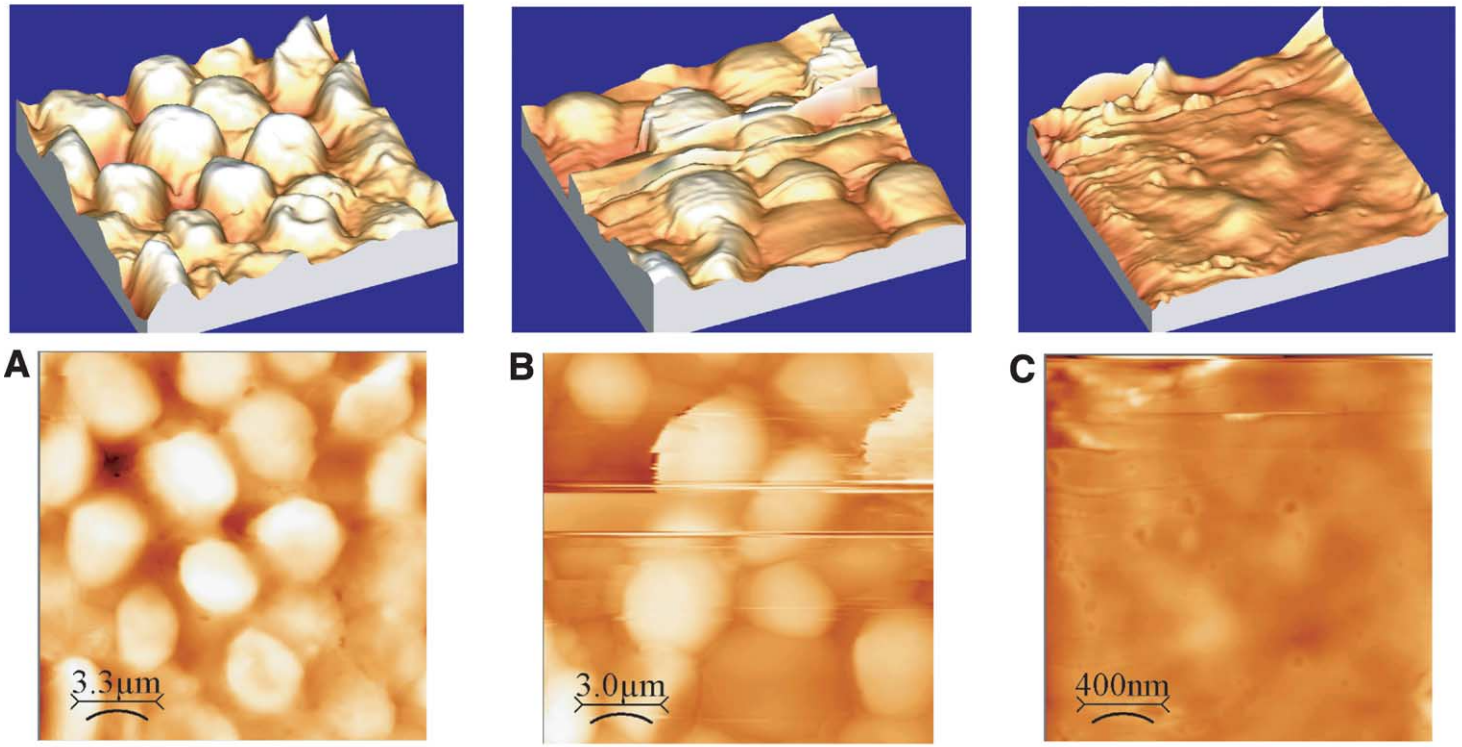


Fig. 1. Atomic force micrographs showing the three- and two-dimensional morphological surface of *S. cerevisiae* cells. A: Control: scanning of the *S. cerevisiae* surface with a common tip (cantilever without nimodipine on the tip). B: Scanning of the *S. cerevisiae* cell surface with a cantilever derivatized with nimodipine (15 μm). C: Scanning of a single *S. cerevisiae* cell surface with a cantilever derivatized with nimodipine (2 μm).

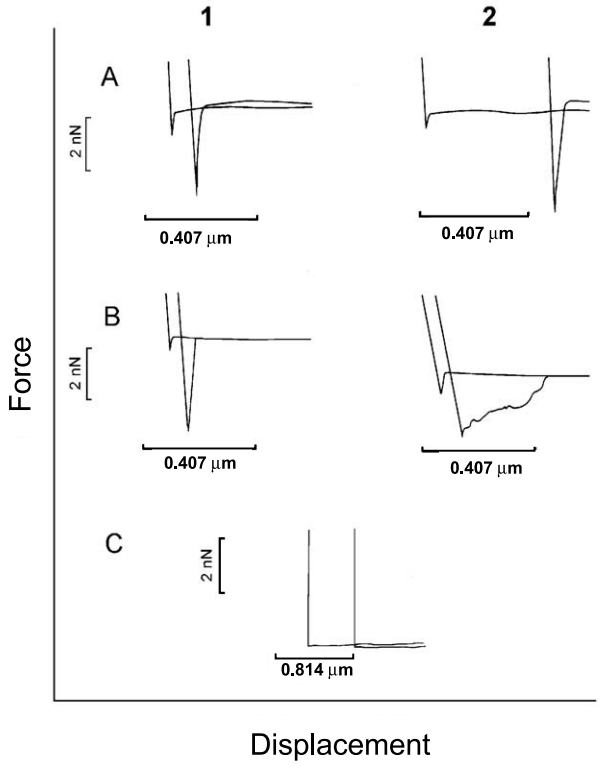


Fig. 2. Cantilever deflection curves on approach and retraction of a nimodipine tip on a *S. cerevisiae* cell surface. A1: Control: a non-modified cantilever (without nimodipine on the tip) on a *S. cerevisiae* surface (outside the pore). A2: Control: a cantilever, without nimodipine on the tip, on a *S. cerevisiae* surface (inside the pore). B1: A nimodipine tip on *S. cerevisiae* (outside the pore). B2: A nimodipine tip on *S. cerevisiae* (inside the pore). C: A nimodipine tip on *S. cerevisiae* (with free nimodipine previously added to the cell surface).

and the free nimodipine molecules added to the cell surface. The same graph was obtained by Florin and collaborators, when a similar experiment was made using avidin immobilized on the cantilever tip and biotinized agarose beads instead of living cells [16].

As it was observed previously [1,2], using a non-modified cantilever, the size of the pores (of the *S. cerevisiae* cells) varies from 50 to 250 nm [1,2]. It seems that this physiological condition is controlled by the cells, occurring normally in the calcium uptake and it is independent of the tip used (non-modified or modified cantilever).

4. Conclusions

Until now, for measuring the binding of a medication with its receptor it has been necessary to use a drug that has tritium (^{3}H) or ^{125}I and that these isotopes have 5 Curie per mmol at least [10]. With this methodology of radioactive drugs the development of trials for receptors of ^{3}H-opioid were possible, which led to the discovery of antialgic natural substances: the endorphins and enkephalins [10].

These trials involving receptor are a simple, fast and reliable method for screening several potential drugs. A drug, for example, which replaces ^{3}H-haloperidol from its membrane receptor can be a good neuroleptic.

Today, this technique can be replaced by a cantilever derivatized with a drug, as described in this paper, with some advantages: (i) with the cantilever derivatized method it is possible to visualize the cells (or the sample) and the area of the drug receptors; (ii) this technique is fast, simple and reliable and radiolabeled drugs (which are expensive and are no easy to be obtained) are not necessary. The cantilever derivatized method uses commercially available drugs or organic molecules with potential pharmacological uses, making possible screening of new pharmaceuticals and new uses for the existing medications.

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